CHAPTER 5

Coordination of Nucleases and Helicases during DNA Replication and Double-strand Break Repair

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5.1 Introduction

Nucleases and helicases are involved in numerous steps in DNA replication and repair. Nucleases act on intermediates in DNA replication created by DNA polymerases (Chapter 4) and helicases (Chapter 3). They can create substrates for repair as in Okazaki fragment processing (OFP) and homologous recombination. They can also create substrates for activation of a checkpoint response, or participate in downregulation of checkpoints. In the special case of telomere replication, they are also involved in essential processing steps (Chapter 8). Nucleases known to act during DNA replication include Dna2, Rad27, Mre11, Sae2, Exo1, RNaseH, Yen1 and Mus81/Mms4. Of these, Dna2, Exo1 and Mre11 are of particular interest because they have been identified as crucial activities that initiate repair of double-strand breaks (DSBs) by homologous recombination and thus form an intrinsic link between DNA

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replication and repair of DSBs derived from replication fork failure. The action of the nucleases is coordinated with those of a number of helicases and is discussed here in the context of a network of their interactions that combine to maintain genome integrity during DNA replication.

Fidelity of DNA synthesis is traditionally defined at the level of polymerization (see Chapter 4). Three major mechanisms prevent errors occurring in the nucleotide code: correct insertion; proofreading; and mismatch repair. However, the helicase/nuclease network (Figure 5.1) identifies a much more complex system for maintaining the fidelity of transmission of a complex genome. While the network reflects the multitasking nature of DNA replication proteins in DNA replication and repair of exogenous damage, it more probably evolved as a highly coordinated system for preventing or resolving damage due to aberrant steps in endogenous processes such as DNA replication. The future goal of studies of these enzymes lies in understanding the biochemical underpinnings of the entire system as an ensemble, rather than of the individual components. Such a goal can only be realized using combined genetic and biochemical approaches. This chapter summarizes the individual activities in the context of networks. The activities that are emphasized are highlighted by red circles in Figure 5.1. While the attempt at mechanistic integration is new, analysis of the literature suggests we already have an informative framework to build upon. To usefully limit the discussion, this review focuses mainly on the *Saccharomyces cerevisiae* orthologues of these conserved activities.

5.2 The Role of Nucleases in Okazaki Fragment Processing

Most central to the DNA replication process (because it is a constitutive component) is the role of helicase/nuclease coordination in Okazaki fragment maturation (see also Chapters 1 and 3). Okazaki fragments are initiated by pol α-primase, which synthesizes an RNA/DNA primer of about 10–30 nucleotides. A polymerase switch to pol δ then occurs (see Chapter 6), and pol δ synthesizes the remaining fragment. When pol δ reaches the 5′ RNA terminus of the previously synthesized Okazaki fragments, the RNA is nucleolytically removed and the two DNA fragments are ligated together (Figure 5.2).

5.2.1 FEN1

The prevalent model for Okazaki fragment processing in eukaryotic chromosomes originally included a single, structure-specific nuclease called FEN1, whose role in primer removal was discovered during reconstitution of SV40 DNA replication *in vitro*.[1] FEN1's primary enzymatic function is that of a structure-specific nuclease that binds to a free 5′ end and tracks along the single strand until it reaches a single-strand/duplex junction where it cleaves the single strand endonucleotytically (Figure 5.2 left panel). It is thus ideally suited to cleave a 5′ single-stranded RNA/DNA flap, created by pol δ strand

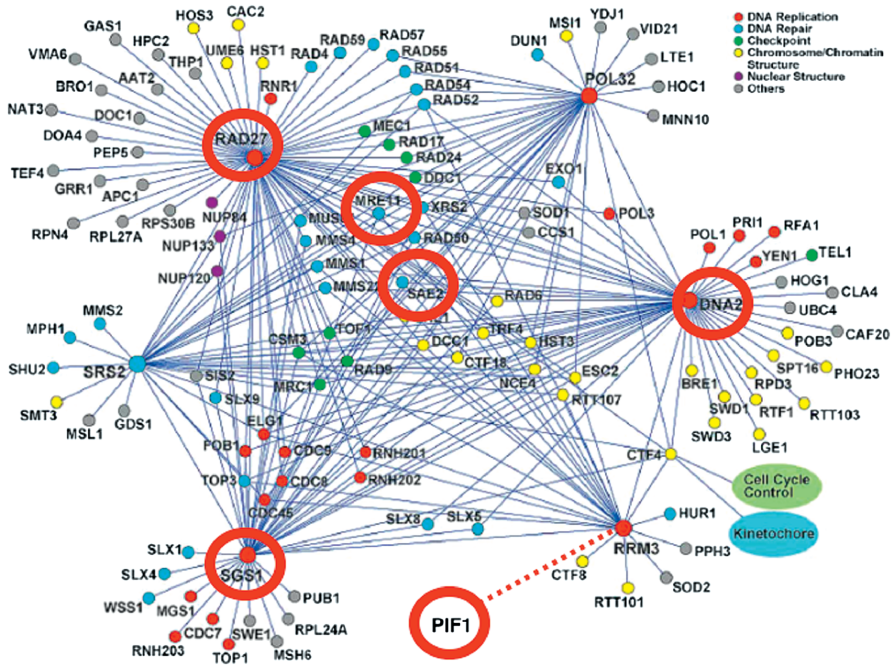


Figure 5.1 **A network for preserving genome stability through the DNA replication fork.** This network was derived from studies in ref. 6; similar but more complex networks are described in refs. 30, 190. *rad27* mutants are synthetically lethal with: helicase mutants *sgs1, srs2, dna2*; replication mutants *pol32, mrc1*; checkpoint mutants *rad9, rad24, rad17*, *mec1, mrc1, tof1, csm3*; nuclease mutants *exo1, rnh202, mus81|mms4;* repair mutants *rad1, rad50,51,52,54,55,57,59* and *sae2;* cohesion mutants *ctf4, ddc1;* and chromatin mutant *asf1*.[147] The list of synthetically lethal interactions with *rad27* mutants is larger than the list of synthetically lethal interactions of any of the following mutants*: dna2, mre11, sgs1, srs2, rrm3, exo1, sae2, yen1*. The large synthetic lethal list suggests that Rad27 (FEN1) is directly involved in DNA replication and not just repair of replication errors. The absence of *rad27* creates double-strand breaks (DBS) requiring the recombinational repair and checkpoint complexes for viability. The synthetic lethality network of *dna2* mutants does not include the checkpoint mutants *rad9, rad24, rad17, mec1*, nor repair mutants *rad51, 55, 57*, but does include all the genes encoding nucleases involved in OFP: *i.e. rad27, exo1, yen1, rnh202*, repair mutants *rad50, rad52*, and *sae2*.[6] Like *dna2* mutants, *mre11* mutants are not synthetically lethal with checkpoint mutants *rad9*, *rad24*, *mec1*, nor repair mutants *rad51*, *rad52*.[191] The remaining nucleases—Sae2, Exo1, Yen1 and RNaseH—form a synthetic lethal network with many fewer nodes[192] than the Rad27, Dna2 or Mre11 subnetworks, although *dna2* is synthetically lethal with all four.[6] We propose that the high node networks based on *rad27*, *mre11* and *dna2* suggest that these nucleases act first at DNA replication and repair intermediates.

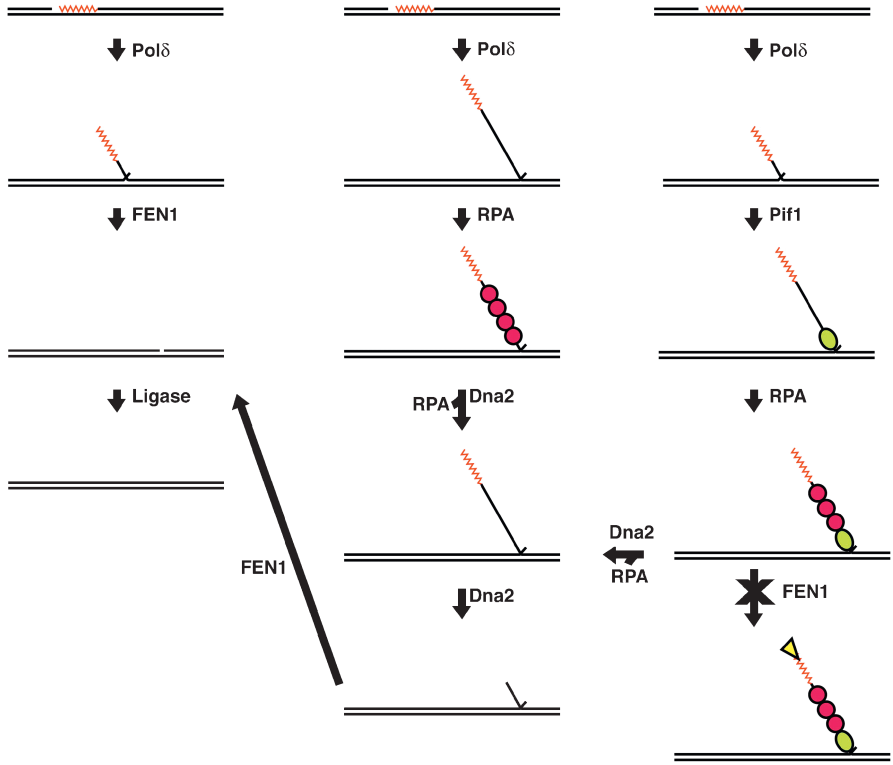


Figure 5.2 **Multiple modes of Okazaki fragment processing (OFP) in eukaryotic cells.** Details are given in the text. The preferred substrate for FEN1 is a double flap as shown: a 5′ flap and a 3′ flap of 1 nucleotide overhang from the upstream fragment.[192,193] An equilibrating flap can occur when both the 5′ flap and the 3′ flap are complementary to the template. Red circles: RPA; green oval, Pif1; yellow triangle, FEN1.

displacement, to form a ligatable nick between adjacent Okazaki fragments (see ref. 2) (Figure 5.2 left panel). In yeast, mutants lacking FEN1 (*i.e. rad27Δ*), are viable but temperature sensitive.[2,3] The viability derives from backup mechanisms, the most direct one consisting of an orthologue of FEN1, the nuclease Exo1 (exonuclease 1). The human counterpart of Exo1 is also a structure-specific nuclease[4] that compensates, albeit inefficiently, for deficiency of both FEN1[5] and Dna2[6] (see Section 5.2.2). There is also an indication that RNaseH can participate in removal of RNA primers in an alternative mode of processing (see Figures 5.1 and 5.2).[6–8] The importance of FEN1 in OFP is highlighted by the finding of very high rates of gross chromosomal rearrangement (GCR) in *rad27Δ* strains, consistent with FEN1 processing the vast majority of Okazaki fragments and of chromosomal breaks occurring in its absence.[2,9]

5.2.2 Dna2

It now appears that the minimal FEN1-mechanism shown in Figure 5.2 (left panel) is not sufficient to maintain a complex genome. With the discovery of Dna2 helicase/nuclease and the fact that Dna2 is an essential protein that interacts with FEN1, the FEN1-alone processing model was expanded to include Dna2.[10–14] Dna2 is an endo/exonuclease that prefers a 5′ flap structure, though it also has limited 3′ to 5′ nuclease activity. The multiple nuclease activities of Dna2 are catalyzed by a single active site homologous to the RecB nuclease of *Escherichia coli*, located in the N-terminal half of the protein.[15–17] Dna2 also has 5′ to 3′ ATP-dependent DNA helicase activity,[10,11,17–19] and the helicase motifs lie in the C-terminal region. Dna2 helicase is a member of a subgroup of superfamily 1 helicases with homology to Upf1 helicase. Interestingly, the other members of the family are RNA helicases involved in modulating translation, while Dna2 is involved in DNA replication and recombination.[20,21] Recently, strand exchange and strand annealing activities have also been observed in Dna2 protein preparations.[22]

The 5′ flap nuclease/helicase activity of Dna2 suggested that it might assist FEN1 in RNA/DNA flap removal. Consistent with this proposal, deletion of *DNA2* results in yeast inviability, and *dna2-1* mutants synthesize only short fragments of DNA under non-permissive conditions.[10] *dna2* mutants are also sensitive to MMS, HU, X-rays and bleomycin.[23] Yeast cells that lack FEN1 and are also mutant for Dna2 (*i.e. dna2-1 rad27Δ*) are inviable. Furthermore, *dna2-1* temperature-sensitive growth is suppressed by overexpression of FEN1, supporting the idea of a two-nuclease processing model for OFP (Figure 5.2 centre).[12]

5.2.3 FEN1, Dna2 and RPA Cooperate in OFP

A specific biochemical model for how Dna2 might stimulate FEN1 in OFP arose initially from another genetic observation, namely that *rfa1*-Y29H, a mutation affecting the single-stranded DNA binding protein subunit, Rpa1, was synthetically lethal with *dna2-157* (Cys1255Tyr mutation in Dna2 helicase motif III).[24] Further analysis showed that this behaviour reflects specific interactions between the two proteins. Almost all *rfa1* mutants mapping to the C-terminal DNA binding domain were also synthetically lethal with *dna2-157*. Conversely, almost all *rfa1* mutants in the N-terminal 17 kDa protein–protein interaction domain were viable in combination with *dna2* mutations. Almost all helicase domain *dna2* mutants are synthetically lethal with *rfa-Y29H* (including *dna2-2* (R1253Q), which is completely viable as a single mutant); in contrast, all *dna2* nuclease domain mutants are viable in combination with the *rfa-Y29H* allele, although some are sick due to low expression. Physical association between Dna2 and Replication Protein A (RPA) was also observed, with the C-terminal Dna2 helicase domain interacting primarily with the C-terminal two-thirds of RPA, but interaction between the N-termini of the two proteins also modulates this C–C interaction.[24] Furthermore, RPA stimulates both the

helicase and the 5′ to 3′ nuclease activities of Dna2.[24–26] On the other hand, RPA inhibits Dna2-dependent cleavage of 3′ single-stranded regions adjacent to a duplex segment.[27] In terms of physiological function, it is probably significant that, in proteomic experiments involving affinity purification of protein complexes, the Dna2 protein always appears in association with RPA, and *vice versa*.[28–30]

The combined nuclease/helicase activities of Dna2 and its interaction with RPA led to a detailed model of how the two-nuclease OFP processing system might function.[26,31] In this model, DNA pol δ was proposed to displace a 5′ RNA/DNA primer flap longer than 30 nucleotides—a length of DNA that binds RPA efficiently (Figure 5.2 centre). Such a structure had been shown to inhibit FEN1.[32,33] Therefore, it was proposed that the RPA-coated flap first recruited Dna2, which cleaved the flap to within five nucleotides of the branchpoint. Short flaps do not bind RPA tightly and thus Dna2 creates an optimal flap substrate for FEN1. In this model, Dna2, since it was an essential gene, was envisaged as having a role at every Okazaki fragment.

5.2.4 DNA Polymerase δ Exonuclease Activity in OFP

A key element of the two nuclease model is that DNA pol δ needs to displace a flap of 30 nucleotides or longer, because shorter flaps would not stably bind RPA and therefore FEN1 alone would be sufficient. Genetic observations, followed by an investigation of the underlying biochemical principles of the reactions, led to a more complete picture of OFP. Pol3-5DV is a mutant deficient in the 3′ to 5′ proofreading activity of pol δ. While there is no severe defect in OFP in either *rad27Δ* or *pol3-5DV* exonuclease-deficient single mutants, the double mutant shows a severe growth inhibition and a mutator phenotype consistent with a defect in OFP.[34] The major class of mutation that arises is long duplications (up to 100 bp flanked by short direct repeats).[34,35] This suggested a mechanism of OFP requiring both the pol δ 3′ to 5′ exonuclease and FEN1, and tight coupling between them.

Pol δ lacking 3′ to 5′ nuclease activity shows intrinsically increased strand displacement compared to wild-type enzyme, and *in vitro* reconstitution reactions mimicking OFP demonstrated a role for the 3′ to 5′ proofreading activity of pol δ in limiting the extent of strand displacement by pol δ.[36–38] When pol δ extends a primer in the presence of PCNA and FEN1, it usually displaces a downstream blocking DNA oligonucleotide (representing an Okazaki fragment) by only one or two nucleotides, not 30 nucleotides. The strand opening creates a 5′ flap that is coordinately cleaved by FEN1 (nick translation) or which re-anneals to the template creating a 3′ flap that is removed by the 3′ exonuclease of pol δ (idling: dNTP to dNMP turnover is not accompanied by net synthesis or degradation).

The DNA at the junction between Okazaki fragments thus cycles between (i) a ligatable nick formed by synthesis by pol δ and reversal by 3′ exo (idling), and (ii) a ligatable nick formed by limited strand opening giving a 2-3

nucleotide flap cleaved by FEN1 (nick translation). The nick translation reaction can remove RNA, and the idling reaction prevents extensive strand displacement. The latter was demonstrated[38] by showing that:

- only if the 3′ to 5′ exo is defective, does pol δ/PCNA extensively displace the downstream oligonucleotide;
- wild-type pol δ/PCNA only opened the downstream oligo by one or two nucleotides, yielding FEN1-mediated cleavage products of that length when FEN1 was present.

Pol δ and FEN1 also function coordinately to nick translate into a blocking oligonucleotide terminated with RNA—a substrate that mimics an Okazaki fragment. In these reactions, FEN1 is thought to be coordinated through its binding to PCNA,[39] with a hinge region of FEN1 permitting it to swing out to optimise nuclease cleavage[40] (see Figure 3.8E).

5.2.5 Dual Mode OFP

Since the intrinsic properties of pol δ/PCNA and FEN1 thus obviated the need for Dna2, the OFP model has been revised to suggest that there are two alternative modes of processing.[38] The predominant mode of Okazaki fragment processing occurs through short flaps and the one-nuclease (FEN1) mechanism (Figure 5.2 left panel). Dna2 participates only in cases where flaps become long—for instance, under conditions where strand displacement is extensive either because FEN1 is inhibited or due to inhibition of the 3′ exonuclease reaction (Figure 5.2). In keeping with the proposal that extensive strand displacement gave rise to the essential role of Dna2, the double mutant *dna2-1 pol3-01* is inviable and overexpression of *DNA2* suppresses *rad27p pol3-5DV rad51* lethality (*pol3-01* and *pol3-5DV* are 3′ exonuclease-defective mutants of DNA pol δ, *dna2-1* is a Dna2 hypomorph, and *rad27p* encodes a FEN1 defective in PCNA interaction but yeast cells with this mutation grow normally[41]). Note that Rad51 is required for repair of double-strand breaks which are assumed to occur in *rad27p pol2-5DV* mutants.[6,37] Furthermore, deletion of Pol32, a subunit of pol δ that is required for efficient strand displacement, suppresses the phenotype of *dna2-1* and *dna2-2* mutants.[6,42] Finally, further biochemical reconstitution studies defined specific conditions—mutations in the relevant proteins, DNA sequence variation, and reaction conditions—that regulated the proportion of long flaps.[43] In other words, the two-nuclease model is not a constitutive feature of OFP, but occurs only under conditions where flaps become long. This raises the question of what specific conditions lead to the requirement for the additional helicase-nuclease, Dna2. A clue to this question came from genetic analysis that revealed that *pif1Δ* suppresses the lethality of *dna2Δ* and the temperature sensitivity of *dna2-1*.[44]

5.2.6 Pif1 Helicase Regulates OFP

Pif1 is a 5′ to 3′ helicase that has the ability to unwind an RNA/DNA duplex, and is a known inhibitor of telomerase.[45–47] Pif1 also has a mitochondrial form that is required for mitochondrial DNA recombination.[44,47,48] Although not essential in *S. cerevisiae*, its orthologue, Pfh1, is essential in *Schizosaccharomyces pombe*.[49–51] In both organisms, Pif1 shows extensive genetic interactions with DNA pol δ and its subunits.[44,49] *dna2Δ pif1Δ* mutants are temperature-sensitive and sensitive to methyl methanesulfonate (MMS), but, unlike *dna2Δ PIF1* mutants, they are resistant to X-rays. The viability of *dna2Δ pif1Δ* mutants suggests that the presence of functional Pif1 makes Dna2 essential. One way Pif1 helicase could do this is to create a structural intermediate that requires processing by Dna2 helicase–nuclease. Long flaps formed by Pif1 in conjunction with pol δ, a reaction suggested by their genetic interactions, could constitute such a structure. Indeed, RPA does not inhibit FEN1 cleavage of the rare long flaps created by pol δ/PCNA/FEN1 during concerted strand displacement and processing on synthetic substrates. However, RPA does inhibit FEN1 cleavage of long flaps created (more efficiently) by pol δ/PCNA/FEN1 upon addition of Pif1 (Figure 5.2 right panel).[52] Thus, biochemically, Pif1 can direct the OFP reaction toward a mechanism that might require Dna2 to stimulate FEN1. This leads to the further speculation that Pif1 helicase may participate normally in flap generation by pol δ, *i.e.* it could be a polymerase accessory protein. This would be in keeping with *in vitro* kinetic studies where the pol δ/PCNA/FEN1 processing reaction occurs more slowly *in vitro* than the deduced rate of OFP *in vivo*, suggesting that some component present *in vivo* is absent in the reconstituted system;[36,37] Pif1 could be the missing component that yields the physiological rate. To date, however, it has not been investigated as to whether Pif1 increases the rate of processing. Genetic evidence for a Pif1/pol δ interaction *in vivo* stems from the observation that a *dna2Δ pif1Δ* double mutant is temperature-sensitive, but addition of a third mutation, *pol32Δ*, suppresses the defect.[6,44] Since the Pol32 subunit of pol δ enhances the ability of pol δ to strand displace, its deletion (*i.e. pol32Δ*) may reduce strand displacement. In addition, *pifΔ* suppresses the cold sensitivity of a *pol32Δ* strain.[44] Although to date biochemical studies support a model whereby Pif1 creates a substrate for Dna2, it is also possible that absence of Dna2 protein leads to structures that are lethal if Pif1 is present.

The studies summarized here suggest that Pif1 may be a component of the replication fork that is required to regulate the kinetics of OFP, in addition to its well-understood role in regulation of telomere length (see Section 5.6 and Chapter 8). The difference in the degree to which Pif1 is required for viability in *S. cerevisiae* and *S. pombe* may derive from the fact that regulatory mechanisms, even kinetic ones, are less strictly conserved than the underlying machinery of DNA replication. Perhaps the genome of *S. cerevisiae* is not as full of sequences that require the additional helicase for rapid RNA primer removal, or perhaps there are additional backup mechanisms of processing in *S. cerevisae* that are not found in *S. pombe*. It will be interesting to elucidate the differences in future studies.

5.2.7 Flap Processing in OFP

Since FEN1 is a tracking enzyme that requires a free 5′ end to enter a flap and track to the branch point, a mechanism must exist for removing RPA and Dna2 from a flap so that FEN1 is not blocked from reaching its substrate. Detailed analysis of the pairwise sequential actions of RPA, Dna2 and FEN1 support a sequential model for Dna2/Rad27 flap processing.[53] FEN1 itself has been shown to dissociate Dna2 that has been prebound to a DNA flap.[53] This reaction uncovered an interesting new bimodal function for Dna2. Dna2 nuclease requires a free 5′ end for activity (tracking mode).[54] However, in the absence of a 5′ end, Dna2 can bind a flap efficiently, even a flap as short as two nucleotides, though it does not cleave (non-tracking mode) (ref. 53; J.A. Stewart, J. L. Campbell and R.A. Bambara, unpublished data). This would predict that Dna2 should inhibit FEN1 because it would block FEN1 tracking. However, FEN1 can dislodge Dna2 bound in either the tracking or non-tracking modes.

To investigate how Dna2 can cleave an RPA-bound flap, Stewart *et al.*[55] used a nuclease-deficient yeast Dna2 protein to show that Dna2 can dissociate RPA from a flap even in the absence of Dna2-dependent cleavage activity. Furthermore, Dna2 tracking activity was not required for RPA removal although it is needed for Dna2 nucleolytic function, providing further evidence that flap cleavage is not required for dissociation of RPA.[54,55] This activity of Dna2 is probably predicated on specific protein–protein interactions, since yeast Dna2 cannot displace human RPA, perhaps explaining why hRPA inhibits yeast Dna2 cleavage. Furthermore, RPA stimulates Dna2 nuclease preferentially on flaps containing secondary structure by aiding in denaturation of intrastrand structures. In many situations in the cell, RPA must be removed to allow access by other enzymes. Thus, Fanning and coworkers[56,57] have suggested that protein–protein interactions cause a change in conformation of RPA to a weakly bound form that can then be easily displaced by other proteins, allowing access to the DNA of further enzymatic activities (see also Chapter 6). Dna2 may interact with RPA to promote its dissociation from DNA and to allow Dna2 binding and DNA degradation. It is therefore very likely that dissociation of RPA from the DNA is an important activity of Dna2 protein.

5.2.8 Dna2 Helicase Activity in OFP

What is the role of the helicase activity of Dna2 that is essential? Biochemical studies indicate that the Dna2 helicase acts in a coordinated fashion with its nuclease on flaps that have secondary structure, to produce a FEN1-cleavable product.[25,58] Note that Dna2 tracks in the 5′ to 3′ direction and cleaves the strand on which it is tracking. The ability to suppress the lethality of deletion of *DNA2* by deletion of *PIF1* has allowed the coordination to be demonstrated *in vivo* as well. *dna2Δ pif1Δ* strains transformed with plasmids expressing *dna2-helicase-plus; nuclease-minus* (H^+N^-) mutants become inviable, while plasmids

expressing *dna2-helicase minus, nuclease minus* (H^-N^-) as well as *dna2- helicase-minus, nuclease-plus* (H^-N^+) plasmids lack toxicity (M. E. Budd and J. L. Campbell, unpublished data). Therefore, Dna2 helicase must be active to generate a requirement for the Dna2 nuclease. Another possible role for the Dna2 helicase/ATPase might be in dislodging RPA (see Section 5.2.7).

5.3 Mismatch Repair in DNA Replication: the Importance of Exo1

Errors occurring during DNA polymerisation include the incorporation of mismatched bases which, if not removed by the nuclease activity of the polymerase itself (see Chapter 4), must be removed following fork passage. Exo1, a $5' \rightarrow 3'$ exonuclease, was initially identified as a mutator in *S. pombe*, suggesting an involvement in mismatch repair.[59] The protein is a member of a family of $5'$ to $3'$ nucleases including Rad27 (FEN1), Rad2 (excision repair endonuclease), Din7 (a mitochondrial enzyme) and putative nuclease Yen1 (which is synthetically lethal with *dna2*). Exo1 interacts physically with Msh2, a protein that recognizes base mismatches and is required for mismatch repair.[5] Analogous to the situation in *S. pombe*, the mutation rate of *exo1* strains of *S. cerevisiae* was significantly increased over wild type, although it was not as high as in *msh2* strains.[5] The mutation rate of *msh2* and *exo1 msh2* strains was the same, suggesting they function in the same pathway.[5] Addition of Exo1 restored mismatch-dependent, bi-directional excision activity to an *in vitro* human cell extract lacking mismatch repair excision activity.[4] The human mismatch repair protein hMutSa (orthologue of Msh2) activated the exonuclease activity of hExo1 in a mismatch dependent manner.[4] These results suggest that mismatch base repair is the primary function of Exo1 in DNA replication, although Exo1 can also function as a backup nuclease in OFP, DSB processing (see Section 5.4.4) and telomere processing (see Section 5.7). This mismatch repair may make a significant contribution to replication fidelity in much the same way as extrinsic proofreading (see Section 4.4.5).

5.4 Nucleases and Helicases in Double-strand Break Repair

During DNA replication, forks may stall at unusual DNA structures such as hairpin loops, fragile sites, replication slow zones, or at lesions resulting from exposure to DNA damaging agents. In particular, attempts to replicate past a single-strand break on the lagging strand template will result in the formation of a double-strand break (DSB)—a highly mutagenic and lethal lesion if not repaired rapidly in an error-free manner. Replication checkpoints are signalling pathways that detect DNA damage (especially DSBs) and signal, through phosphorylation of adapter (*e.g.* ATM/R) and effector (*e.g.* MRX/N) molecules, to cause replication arrest and trigger DSB repair (reviewed in ref. 60).

The Mre11/Rad50/Xrs2 (MRX) complex plays a critical role in DNA damage checkpoint pathways and in effecting DNA repair (especially of DSBs) during DNA replication, as suggested by the slow growth of *mre11Δ* mutants and synthetic lethality networks such as that shown in Figure 5.1. The MRX complex in yeast (MRN in other systems) is composed of three main protein components: Mre11, Rad50 and Xrs2 (the mammalian orthologue of Xrs2 is Nbs1, mutated in Nijmegen breakage syndrome). Consistent with an important role in DNA replication, repair and genome stability, *mre11*, *rad50,* and *xrs2* mutants are sensitive to X-rays, MMS, ultraviolet (UV) radiation and hydroxyurea (HU),[i] and are slow growing. It has recently become apparent that Sae2 is a nuclease that acts synergistically with Mre11, and *sae2* mutants show phenotypes similar to those of certain *rad50* and *mre11* mutants. Sae2 is therefore discussed in conjunction with Mre11.

5.4.1 Mre11

Mre11 provides the active nuclease of the MRX complex, showing 3′ to 5′ exonuclease activity on double-strand substrates and endonuclease activity on DNA hairpins.[61–63] The Mre11 N-terminus contains five phosphodiesterase motifs also found in *E. coli* sbcD exonuclease.[64,65] The enzyme requires Mn^{2+} for activity rather than Mg^{2+}, since the phosphodiesterase motifs contain four histidine residues which orient Mn^{2+} but will not bind Mg^{2+} [66] (see Figure 5.3A). On nuclease cleavage of DNA, histidine 125 (H125) of Mre11 protonates the leaving 3′ hydroxyl (OH) of the penultimate deoxyribose sugar; consistent with this, mutation of H125 inactivates the nuclease.[66] Four nuclease-deficient *mre11* mutants fail to bind Nbs1 (the mammalian orthologue of Xrs2) in the hMRN complex, suggesting that Nbs1 probably binds near the nuclease active site of Mre11.[67] Biochemically, Xrs2/Nbs1 is unlikely to have catalytic activity, but it probably mediates interaction with other factors important for checkpoint signalling such as BRCA1, perhaps through its forkhead associated domain (FHA) which acts as a phosphopeptide binding motif.[68]

5.4.2 Rad50

The Rad50 component of the MRX/N complex is an ATP binding protein in which the Walker A and Walter B motifs are separated by 600–900 residue heptad repeats which create a coiled coil.[69,70] Rad50 is a member of the Structural Maintenance of Chromosome family, which includes cohesins Smc1 and Smc3 (see Chapter 9), and *E. coli* sbcC. The Rad50 C terminal domain shows homology to ATP binding cassette (ABC) type ATPases, which include the cystic fibrosis transmembrane conductance regulator (CFTR).

Rad50 binding to DNA is dependent on ATP.[71] ATP binds to the P loop in the N-terminus and a conserved region in the C-terminus that performs a

[i] An agent that disrupts nucleotide pool sizes leading to replication fork arrest and induction of the S phase checkpoint.

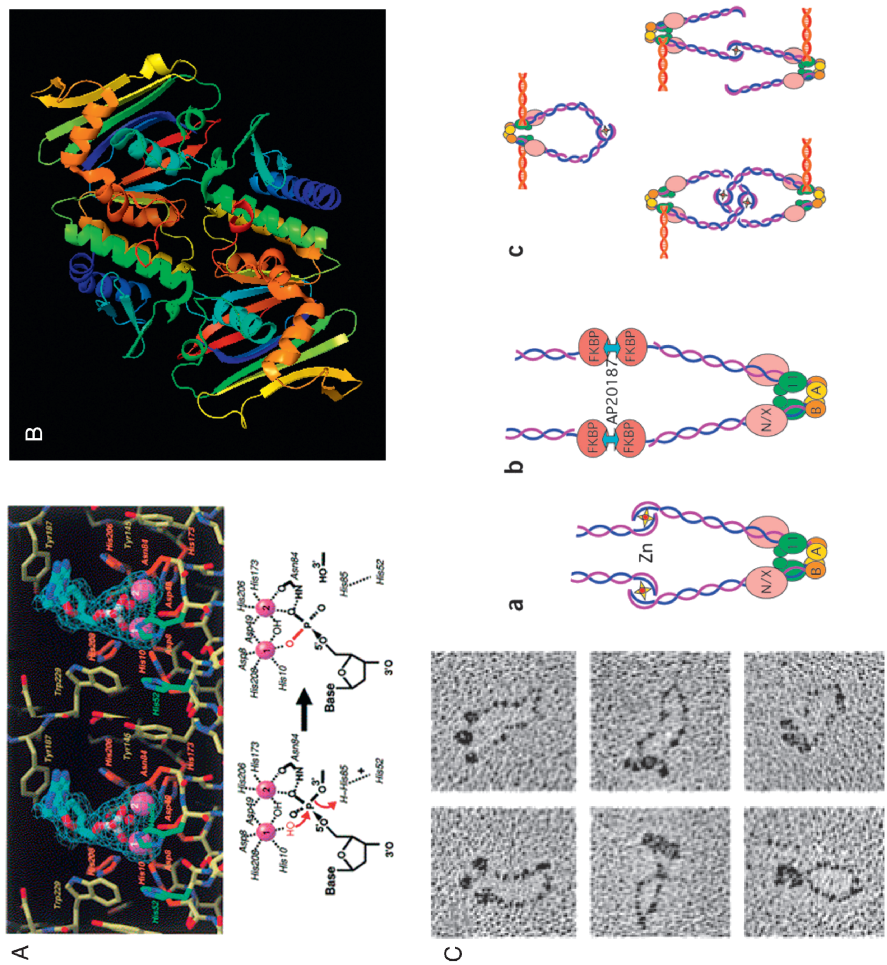
similar function as region VI in the single subunit helicases, or the arginine finger in hexameric helicases[66,69,72] (see Figure 5.3B). This region is called the signature motif.[73] Mutation of a conserved serine in this signature motif abolishes Rad50 ATP binding. (When the same mutation is made in the CFTR protein, the result is cystic fibrosis.[73]) *rad50* strains with mutations in the Walker A motif or signature motif have the same sensitivity to DNA damaging agents as *rad50Δ* mutants,[73,74] reinforcing the importance of nucleotide binding for Rad50 function. Rad50 ATP binding results in dimerization[69,75] (see Figure 5.3C), and this dimerization leads to enhanced DNA binding by formation of a positively charged surface at the dimer interface. It does this since ATP binding to Rad50 rotates the N- and C-terminal subunits so that the N-terminal DNA binding site of Rad50 aligns with Mre11 bound to the first 40 residues of the coiled coil region in the C-terminus of Rad50 exiting the ATP binding domain[66,76] (see Figures 5.3B and 5.3D). This creates a coupled DNA binding surface.[69]

An additional ramification of Rad50 biochemistry is the demonstration that Rad50 has adenylate kinase activity (*i.e.* it catalyses formation of 2ADP from ATP and AMP). An inhibitor of the adenylate kinase activity of Rad50, Ap_5A, blocks the nonhomologous end joining (NHEJ) reaction mediated by scMre11/Rad50 (scMR), at least in *Xenopus* egg extracts, but has no effect on its ATPase activity nor on the endonuclease activity of hsMRN.[77] (Note that in yeast, Ku-mediated NHEJ is a relatively minor pathway for DSB repair.)

5.4.3 MRX/N Unwinding Creates a Substrate for MRX/N Nuclease Cleavage

Although mammalian MRN does not possess processive helicase activity, a weak ATP-dependent unwinding activity is present.[67] When MRN is assayed in the absence of ATP on an oligonucleotide that forms a hairpin at one end and a protruding single-stranded 3′ region at the other end, cleavage occurs at the hairpin loop at a site where duplex and single-stranded DNA meet. However, in the presence of ATP, numerous cleavages are observed in the 3′ overhang, and these extend into the duplex region in the 3′ to 5′ direction.[67] These cleavages are dependent on the Nbs1 subunit.[67] MRN unwinding activity thus appears to be creating a substrate for its own nuclease. Junctions between single-stranded and double-stranded DNA are hotspots for cutting by yeast MR and MRX/N in the presence of ATP.[62] This cleavage is also dependent on Nbs1, but nuclease cleavage of 3′ overhangs by MRN is inhibited by the addition of RPA or KU.[67]

In *Xenopus* egg extracts, the MRN complex has recently been shown to process DSBs into short single-stranded DNA oligonucleotides of 4–12 bp, which are bound to MRN. The oligonucleotide-bound MRN then activates the checkpoint kinase ATM, as assayed by S1981 phosphorylation.[78] However, this mechanism probably does not function in *S. cerevisiae,* since a nuclease-minus MRX can activate the DNA damage checkpoint, although deactivation is delayed. Moreover, yeast MRX is thought only to activate the DNA damage checkpoint when bound to chromosomes (see below).


A
B
C
a
b
c
Zn
N/X
B
A
FKBP
AP20187

The paradox of the biochemistry of the Mre11 complex is that the enzyme has 3′ to 5′ nuclease activity, but resection of DSBs during double-strand break repair *in vivo* is in the 5′ to 3′ direction. In fact, 3′ ends are stable for up to four hours *in vivo*,[79] reflecting earlier observations of extensive degradation of chromosomes (presumably both 3′ and 5′ ends) at 3.5 hours after X-irradiation of a *rad52* strain.[80] One attractive proposal to reconcile a role for MRN in generating the 3′ ends in DSB repair with its *in vitro* activities is based on the idea that, as MRX unwinds the end of a DSB, the single-stranded DNA forms hairpin loops in the 5′ strand that are cleaved to create a 3′ overhang.[62] Alternatively, other nucleases such as Exo1, Dna2, Sae2 or Rad27 may be associated with MRX and cleave DNA in the 5′ to 3′ direction, revealing a 3′ single strand. Analyzing the phenotypes of Mre11 nuclease defective (*mre11-nd*)

Figure 5.3 **Structural analysis of critical components of the MRN DSB repair complex.** (**A**) Mre11: This crystal structure of *Pyrococcus furiosus* Mre11 shows dimerization of the single active nuclease site of the Mre11 protein bound to Mn^{2+} (pink balls) and to the reaction product, dAMP (red and white stick figure). One active site unites all of the multiple nucleolytic activities of Mre11 in a single mechanism shown schematically below the crystal structure. Reprinted from: K. P. Hopfner, A. Karcher, L. Craig, T. T. Woo, J. P. Carney and J. A. Tainer, Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase, Cell, **105**, 473–485, copyright (2001) with permission from Elsevier and the authors.[66] (**B**) Rad50 ATPase: ATP-free catalytic domain dimer of Rad50 ABC-ATPase from *Pyrococcus furiosus*. Binding of ATP promotes dimerization, and hydrolysis promotes release of DNA and dimer disassembly. Figure generated in MacPyMOL (http://delsci.com/macpymol/) from pdb data 1US8.[69] (**C**) Electron microscopic imaging of *S. cerevisiae* Rad50/Mre11 dimers demonstrating the coiled-coil and flexible hinge region that allows the two active sites to come together. Reprinted with permission from: D. E. Anderson, K. M. Trujillo, P. Sung and H. P. Erickson, Structure of the Rad50. Mre11 DNA repair complex from *Saccharomyces cerevisiae* by electron microscopy, *J. Biol. Chem.*, 2001, **276**, 37027–37033, copyright (2001) by the American Society for Biochemistry and Molecular Biology.[75] (**D**) Rad50 dimerization: (a) Schematic of the Rad50 zinc binding hook domain at the tip of the coiled-coil. This hook also constitutes the hinge region that allows formation of the Rad50 coiled-coil within the MRX/N complex; (b) An experimental system for dissection the contribution of the hook to dimerization and coiled-coil formation described in ref. 76; (c) Possible intramolecular and intermolecular structures mediated by the hook. Mre11 (green), is shown as a dimer binding between the Rad50 catalytic domains as suggested by electron microscopy; Xrs2/Nbs1 (pink); Rad50 (coil). The yellow A and orange B discs show the location of the Rad50 Walker A and Walker B motifs, respectively. DNA, shown as an orange helix, indicates possible binding modes for DNA. These models suggest how the MRX complex might coordinate the two ends of a DSB. Reprinted by permission from Macmillan Publishers Ltd: M. Lichten, Rad50 connects by hook or by crook, *Nature Structure Molecular Biology*, 2005, **12**, 392–393, copyright (2005).[76]

mutants sheds light on the question whether other nucleases can compensate for Mre11.

Two mutants of particular interest are Mre11 H125N and D56N. These amino acids fall in the conserved phosphodiesterase motif and the mutants have no endonuclease activity *in vitro*;[81] however, they are able to form a complex with Rad50 and Xrs2. Such nuclease-deficient (*nd*) mutants of Mre11 (*i.e. mre11-H125N* and *mre11-D56N*) are much more resistant to X-rays and MMS than strains lacking Mre11 (*i.e. mre11Δ*), although more sensitive than *MRE11* strains,[82,83] and DNA ends at multiple HO-induced breaks are resected at the same rate and extent in *mre11-nd* strains as in MRN^{+}.[84] Similarly in meiosis, a residual ATP-dependent DNA unwinding activity of MRX in the *mre11-nd* mutant appears able to create a substrate on which alternative nucleases can act and process DNA ends.[81,85–87] Taken together, these data suggest that other nucleases can compensate for lack of nuclease activity of Mre11, though not for the complete absence of the Mre11 protein.

5.4.4 Dna2 and Exo1 Can Compensate for Mre11 Nuclease in DSB Repair

Exo1 is one nuclease that can compensate for the absence of Mre11. *exo1Δ mre11Δ* and *exo1Δmre11-nd* strains are viable but slightly more sensitive to X-rays than *mre11Δ* and *mre11-nd* mutants, and have a more significant defect in 5′ to 3′ degradation at a DSB (induced by HO endonuclease) than *mre11Δ* strains.[82] Overproduction of Exo1 increases the X-ray survival of an *mre11Δ* and *rad50* strain and the MMS survival of an *mre11Δ* strain, and restores 5′ to 3′ resection in *mre11Δ* strains.[82,88] The DNA damage resistant phenotype of the *mre11-nd exo1Δ* strains suggests that Exo1 is not the only enzyme that can replace Mre11 nuclease activity, but clearly Exo1 can compensate for repair in an *mre11Δ* strain if overproduced. However, a genetic assay that measures actual repair, namely mating type switching after HO cutting, shows that *mre11-H125N exo1Δ* strains are just as proficient in repair as *MRE11 EXO1* strains.[82] This analysis suggests that yet another nuclease must compensate for Mre11 and Exo1 in repair of DSBs. The demonstration that *dna2Δ pif1Δ* mutants are viable has enabled the clear demonstration of a role for Dna2 nuclease in DSB repair. *dna2* point mutants are sensitive to X-rays;[23] however, *dna2Δ pif1Δ* mutants, while sensitive to MMS, are resistant to X-rays. *dna2Δ pif1Δ mre11-nd* are as sensitive to X-rays as *mre11Δ* strains and also grow more slowly than comparable *dna2Δ pif1Δ* mutants. These experiments indicate a requirement for the Mre11 nuclease in X-ray repair if the Dna2 nuclease is missing, and *vice versa*.[89] Remarkably, either Mre11 nuclease or Dna2 nuclease can function in repair, since the *dna2Δ pif1Δ mre11-nd* repair deficiency is complemented by either the *DNA2* or the *MRE11* gene.[89] Further recent studies show that Dna2 is specifically involved in 5′ to 3′ resection of a DSB and that it acts in conjunction with Sgs1 helicase (see Section 5.5) during repair by single-strand annealing of repeats.[90,91] Both of these latter two studies also

suggest that Mre11 and Sae2 act earlier than Dna2 and Sgs1 and implicate Exo1 as an alternative to Dna2—at least where extensive resection is required, as in single-strand annealing. Dna2 and a RecQ helicase also function in conjunction with Mre11 in a single-strand annealing reaction in *Xenopus* extracts.[92] Together, Mre11 and Dna2 nucleases seem to perform the major resection required for homology based double-strand break repair.

Mre11 nuclease/helicase interaction differs from that of Dna2 nuclease/helicase. The helicase of Dna2 creates a substrate for the nuclease, which cuts the strand on which it is translocating.[17,58] Dna2 nuclease, however, is the only nuclease that can process substrates created by Dna2 helicase *in vivo*, since Dna2 with defective nuclease supports viability in the absence but not in the presence of its intrinsic helicase (in a *PIF1* deletion, see above).

5.4.5 MRN Recruits Effector Proteins to a DSB

Although Dna2 nuclease can compensate for the absence of Mre11 nuclease activity, Dna2 nuclease cannot compensate for the complete absence of Mre11 protein (*dna2Δ mre11Δ pif1Δ* is inviable).[6] Thus Mre11 has additional functions, perhaps in mediating interaction between the two ends of a DSB.

The relative importance of the various nucleases and helicases in effecting repair at DSBs is suggested by their order of arrival at a DSB and their mutual dependency. These same events initiate a cell cycle checkpoint. The Mre11 nuclease is the first protein detected at a DSB, as measured using real-time observation of fluorescently tagged proteins in living cells,[93] and initiates the DNA damage checkpoint signalling pathway, probably by resecting the break to reveal a single stranded region of DNA which is then bound by RPA, followed by the proteins Tel1, Mec1/Ddc2, Ddc1 and Rad52.[93] The association of the homologous recombination proteins is next: Rad51 depends on Rad52, and the association of Rad55 and Rad57 depends on Rad51.[93] Rad52 associates with breaks after Mre11 has dissociated, suggesting that the binding of Rad52 and Mre11 are mutually exclusive (Figure 5.4).[94] In *mre11-nd* and *sae2Δ* strains, the disappearance of Mre11 foci is delayed; the appearance of Rad52 foci is also significantly delayed.[93] This suggests there is delayed processing of DSBs in *mre11-nd* and *sae2Δ* mutants.

Both Mec1 and Tel1 are homologous S/TQ kinases in the DNA damage signalling pathway. Mec1 is required for DNA damage checkpoint signalling and phosphorylates numerous proteins involved in repair including Rad9, Rad55 and Sae2, while Tel1 functions mainly at telomeres but can compensate for the absence of Mec1 in the DNA damage response if overproduced (*tel1Δ* strains are not sensitive to DNA damaging agents).[95–98] Both Mec1/Ddc2 and Rad52 bind to RPA, and it is their association with RPA that targets these proteins to resected DSBs. Mec1 phosphorylates the mediators Rad9 and Mrc1, and the main effector checkpoint kinase, Rad53, resulting in activation.[95–98]

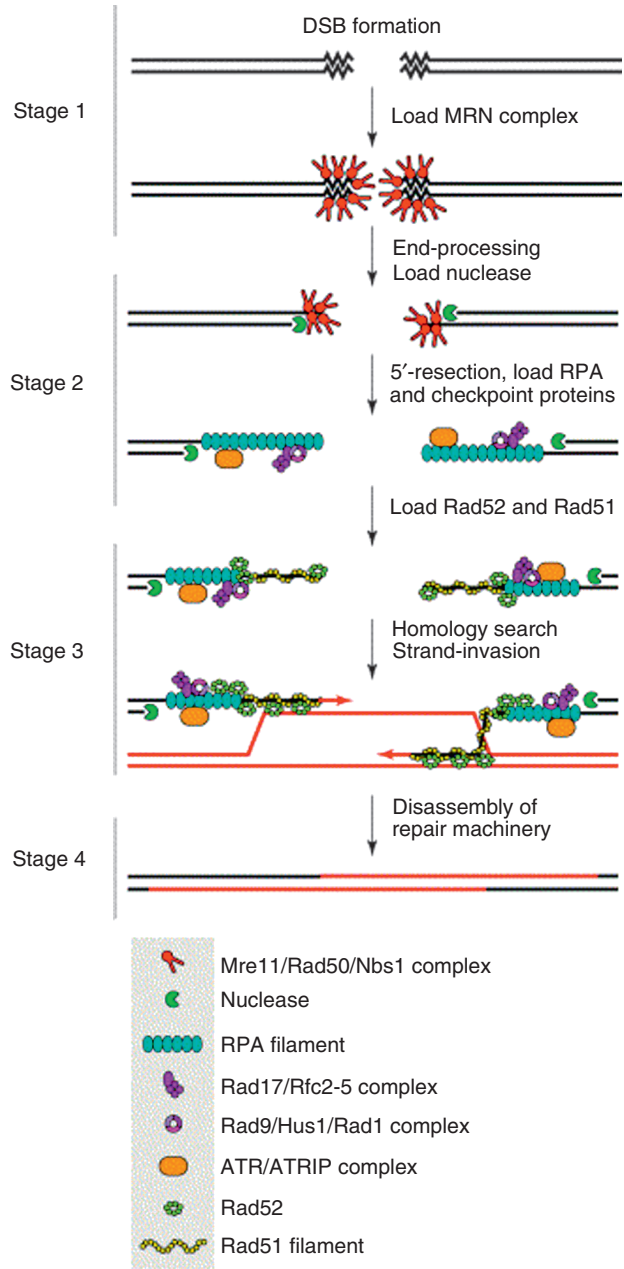


Figure 5.4 **Order of assembly of components at DSBs.** After association of the MRN complex with a DSB, Dna2 and Sgs1 or Exo1 helicases and nucleases may associate and resect 5′ ends, leaving a 3′ single-stranded tail for strand invasion in the strand exchange steps (see text). This stage of DSB repair is represented in the figure by 'nuclease' since the steps have not yet been clearly defined and the specific enzymes involved in specific types of DSB damage remain to be clarified. The remaining stages are described in Section 5.4.5. Reprinted from: M. Lisby and R. Rothstein, DNA damage checkpoint and repair centers, *Current Opinion in Cell Biology*, 2004, **16**, 328–334, copyright (2004), with permission from Elsevier.[94]

In G1 arrested cells, phosphorylation of Rad53 after γ-irradiation is absolutely dependent on MRX.[99] This is sometimes referred to as the MRN-Tel1 checkpoint. Cells of cancer patients with a mutation in either Nbs1 or hMre11 exhibit radiation-resistant DNA synthesis (a phenotype of checkpoint failure) analogous to cells from patients with mutations in ATM (orthologue of Tel1),[100,101] suggesting that the checkpoint role of MRN is conserved from yeast to man. Since RPA recruits Mec1, one expects that Mec1 binding to a DSB would depend on MRX or another resecting nuclease that creates the single-stranded DNA site for RPA binding. In keeping with this suggestion, binding of Mec1 at an HO nuclease-induced break is reduced in an *xrs2Δ* strain, and abolished in an *xrs2 exo1* mutant.[102] After UV treatment (which creates 6-4 photoproducts and pyrimidine dimers that affect usually just one DNA strand of the duplex), MRX is not required for Rad53 phosphorylation, but phosphorylation is nearly blocked in an *xrs2Δ exo1Δ* strain[102] and completely abolished in *mre11Δ exo1Δ* mutants on phleomycin treatment,[102] an agent that leads to DSB formation. Moreover, efficient phosphorylation of Rad53 after low dose (20 mM) treatment of yeast with hydroxyurea is dependent on MRX, although this is not the case after high dose HU treatment (200 mM).[103] DNA replication in the presence of low doses of HU may create DSBs at collapsed replication forks, which require MRX to initiate checkpoint signalling, while high doses of HU may not allow sufficient DNA replication to give rise to DSBs. This idea is supported by the observation that after high dose HU treatment of yeast (100 mM), neither Mre11 foci nor Rad52 foci are observed. However, Mre11 foci are detected in *mec1Δ* strains treated with 100mM HU.[93] Therefore, the absence of DSBs in the presence of high dose HU depends on the presence of a functional Mec1 signalling pathway, suggesting that some HU-induced damage is occurring and that this damage, in the absence of the checkpoint, is converted to DSBs. Mec1 may inhibit a nuclease or other set of events that transforms a stalled fork into a broken fork.

5.4.6 Role of Mre11 and Sae2 in Downregulating the Damage Checkpoint

Mre11 is not only involved in activating the DNA damage checkpoint, but it is also required for checkpoint downregulation, as is Sae2. After an HO-induced DSB, Rad53 phosphorylation persists much longer in a *sae2Δ* mutant than in an *SAE2* strain. The phosphorylation of Rad53 after HO cutting also persists much longer in *rad50S*, *mre11-H125N* and *mre11-D56N* mutants[104] compared with MRX^+ strains. The defective dephosphorylation of Rad53 in *sae2Δ*, *rad50S* and *mre11-nd* mutants suggests that processing and repair of DSBs is significantly delayed if the MRX complex has a defect in nuclease activity. Mre11 foci form normally but persist much longer, and the appearance of Rad52 foci is significantly delayed in *sae2Δ* and *mre11-nd* mutants, presumably due to delayed processing of the DSB.[93] Thus, the nuclease activity of Mre11 and Sae2 is not required to activate the checkpoint, but it is required to

deactivate it. The ATP-dependent DNA unwinding activity of Rad50 may reveal the single-stranded DNA required to initiate the DNA damage checkpoint in the absence of these nucleases.[105] This suggests a model in which Sae2 is involved in processing the DSB together with Mre11, therefore allowing both repair and checkpoint inactivation to proceed quickly. However, this would be an oversimplification, because overproduction of Sae2 blocks the initiation of the DNA damage checkpoint, Mre11 focus formation and Rad53 phosphorylation after DSB formation (by HO cutting) in spite of the normal generation of resected, single-stranded DNA, the presumed signal for checkpoint activation.[104] A more complete hypothesis to explain these findings is that Sae2 may displace Mre11 from DSBs, and when overproduced, it rapidly disassembles Mre11 bound to DSBs, preventing checkpoint activation. Additionally, the off-rate of MRX from DNA might be significantly increased when Mre11 is acting as a nuclease, explaining the persistence of Mre11-nd foci after DSB induction.

Not only does Sae2 inhibit the DNA damage checkpoint, but Sae2 is also regulated by the DNA damage checkpoint. Sae2 is phosphorylated by Mec1 and Tel1 during S phase and after treatment with HU, bleomycin and MMS;[97] treatments that cause, respectively, replication fork arrest, DSBs or mismatches. This phosphorylation may be involved in activation of Sae2 repair activity, since mutations in the S/TQ putative Mec1/Tel1 phosphorylation sites of Sae2, *sae2(1-9)*, renders cells as sensitive to MMS as does the *sae2Δ* mutation.[97] If Sae2 is activated by Mec1 phosphorylation and if activated Sae2 downregulates the checkpoint, then by phosphorylating Sae2, Mec1 is initiating the downregulation of the DNA damage checkpoint. This model suggests that Mec1 is both an activator and later repressor of the DNA damage checkpoint; it is supported by the observation that, in the s*ae2(1-9)* mutant, checkpoint downregulation after UV is significantly delayed. Therefore Mec1 is not acting as a repressor of the checkpoint.[97]

With the DNA damage checkpoint hyperactivated in a *sae2Δ* or *mre11-nd* mutant, one might expect that the complementary Tel1 pathway would be hyperactivated. In keeping with this suggestion, both *sae2* and *rad50S* mutants do suppress the MMS sensitivity of *mec1* mutants in a $TEL1^{+}$ background, and this suppression is absent in a *tel1Δ mec1* mutant.[106] These observations may be explained by the fact that MRX and Tel1 can interact physically, independently of Mec1; Tel1 binds to the C terminus of Xrs2 and is targeted to DSBs by Xrs2.[107]

Xrs2 is thus required for activation of the Tel1 DNA damage checkpoint pathway. To examine the phenotype of disrupting the Xrs2/Tel1 interaction, Mec1 function had to be inactivated, since *MEC1 tel1Δ* strains are resistant to DNA damaging agents. *mec1-81* is a hypomorphic allele of *MEC1* that is sensitive to UV and phleomycin but which, unlike *mec1Δ*, does not result in a proliferative defect in a *tel1Δ* background.[107] *mec1-81 xrs2CTD* (C-terminal deletion that does not bind Tel1) is more sensitive to phleomycin and UV than a *mec1-81* strain, and is as sensitive as a *mec1-81 tel1Δ* strain,[107] showing that *xr2s-CTD* and *tel1* mutants have the same phenotype and may participate in the same pathway. After HO cutting, Tel1 associates with sites near the DSB in

a reaction dependent on a physical interaction between Tel1 and the C-terminal domain of Xrs2.[107] The amount of Tel1 associated with a DSB is the same in a *SAE2* and *sae2Δ* strain. Therefore, enhanced Tel1 signalling in a *sae2Δ* strain probably does not result from more Tel1 binding. Instead, an altered MRX/Tel1 physical interaction may be responsible.

5.4.7 Other Nucleases in Checkpoint Regulation

Do other nucleases play a role in checkpoint regulation? For instance, the synthetic lethality of *mre11Δ* and *dna2Δ* suggests that Dna2 nuclease plays a role in activation of Rad53 after DNA damage, but this has not been formally tested. Rad53 is highly phosphorylated in *dna2-1* mutants, even at the permissive temperature and in the absence of exogenous DNA damage, presumably due to activation of the checkpoint by elongated flaps arising from defective OFP.[44] Deletion of *PIF1* suppresses the endogenous Rad53 phosphorylation in the *dna2-1* and *dna2Δ* mutants. Whether there is any defect in Rad53 phosphorylation in *dna2Δ pif1Δ* mutants after induced DNA damage is not known and whether *dna2Δ pif1Δ mre11-nd* mutants, which are viable but X-ray sensitive, show defects in checkpoint signalling is also unknown. *exo1Δ dna2Δ pif1Δ* mutants are inviable, so examining compensatory interactions of Dna2 and Exo1 is difficult. *sae2Δ dna2Δ pif1Δ* is also inviable, so how Dna2 compensates for the absence of Sae2 is also unknown.

5.5 Repair of Stalled Replication Forks

5.5.1 RecQ Helicases and Nucleases

The single RecQ helicase in *S. cerevisiae*, Sgs1, is homologous to Bloom syndrome (BS) helicase (BLM) and Werner syndrome (WS) helicase/nuclease (WRN) in man, mutations of which predispose to cancer and/or premature ageing (see Chapter 3). In both WS and BS, rates of DNA replication are reduced and illegitimate DNA recombination is greatly elevated,[108–110] with high levels of sister chromatid exchange (SCE) and abnormal processing of DNA replication intermediates as defining phenotypes of BS cells[111,112] compared with replication fork asymmetry and premature replicative senescence in WS.[113,114] Like BS cells, s*gs1Δ* mutants have high levels of recombination between chromosomal repeated DNA elements,[115,116] and Sgs1, like BLM and WRN, is a 3′ to 5′ helicase that unwinds DNA forked structures, Holliday junctions, G4 DNA and single-stranded/double-stranded junctions with a 3′ overhang.[117] *Xenopus* egg extracts depleted of BLM have a similar efficiency of DNA replication as undepleted extracts; however the resulting replicated DNA has chromosomal breaks, although an insufficient number to activate the S phase checkpoint.[118] Depletion of the WRN orthologue, FFA1, has a minimal effect on replication rates in *Xenopus* extracts.[119] In yeast, Sgs1 colocalizes with DNA replication forks during S phase,[120] and both BLM and WRN can be

observed in human cells at nuclear foci representing sites of DNA replication.[113,121,122]

Suppression of yeast replication-defective nuclease mutants by the human BLM and WRN genes has suggested one mechanism by which BLM and WRN may function in DNA replication. Overproduction of hBLM suppresses the temperature sensitivity of *dna2-1* mutants[123] and partially suppresses the temperature sensitivity of a *dna2Δ pif1Δ rpd3Δ* triple mutant strain (M. E. Budd and J. L. Campbell, unpublished data). Similarly, WRN helicase overexpression also suppresses *dna2-1* temperature sensitivity, and a domain C-terminal to the helicase domain is sufficient for this suppression.[124] Since Rad27 (FEN1) overproduction can also suppress the temperature sensitivity of *dna2-1* cells, the most likely mechanism of BLM/WRN suppression is stimulation of the nuclease activity of FEN1. Consistent with this, biochemical studies have shown that BLM and WRN stimulate the flap endonuclease activity of human FEN1, independently of their helicase activity, but requiring the conserved, winged helix RQC (RecQ C terminal) domain.[125,126] The RQC domain of BLM, which lies just C-terminal of the conserved RecQ helicase domain, is a DNA interaction domain; it binds forked structures and G4 DNA, but not Holliday structures.[127] *sgs1* mutants with deletions into the RQC domain are sensitive to MMS and exhibit hyper-recombination between repeated chromosomal sequences, phenotypes characteristic of OFP defects.[128] BLM with a helicase defect suppresses the growth defect of *dna2-1* mutants, but much less efficiently than wild-type BLM, suggesting that the helicase, in addition to the RQC domain, is required for maximal stimulation of FEN1.[123]

If the RQC domain is sufficient for stimulation of FEN1 nuclease on flaps, then why does helicase-proficient BLM suppress *dna2-1* more effectively than helicase-deficient BLM? The Bambara group showed that ATP is required for maximal BLM stimulation of FEN1 nuclease if the flap has secondary structure (such as a fold back, triplet repeat sequence or bubble),[129] whereas if the flap has no secondary structure, then BLM stimulates FEN1 cleavage as efficiently in the absence of ATP (*i.e.* no helicase activity) as in the presence of ATP (where helicase activity is supported). *In vitro* studies also showed that BLM can stimulate disruption of synthetic recombination-like (strand invasion) intermediates formed by 5′ flaps, such as might arise during aberrant OFP, and thereby indirectly stimulate FEN1 flap cleavage.[130] This reaction occurs either in the presence or absence of ATP. In the presence of ATP, BLM may unwind the strand-switched intermediate, while in the absence of ATP, BLM may use its strand annealing and strand exchange activities to resolve Okazaki fragment intermediates that become involved in aberrant recombination structures, converting them back into FEN1 substrates.[130] The key reaction is conversion of a 5′ flap from a duplex structure to a single-strand required for FEN1 loading and tracking.

sgs1 mutants are synthetically lethal with mutants in genes encoding OFP nucleases, Rad27, Dna2 and RNaseH, and also with the Mus81/Mms4 nuclease. The synthetic lethality of *dna2-2* and *sgs1* is only weakly suppressed by a *rad51* mutation (Rad51 coats DNA to form a large nucleoprotein filament

necessary for homologous recombination at Holliday junctions, see Figure 5.4), arguing that most of the lethal damage results from DNA replication defects rather than toxic intermediates potentially formed during recombinational repair of stalled forks. Interestingly, the *dna2-2 sgs1* synthetic lethality/sickness is suppressed by the *fob1* mutation.[131] Fob1 is the rDNA replication fork barrier (RFB) binding protein and is required for replication fork stalling in the ribosomal DNA. Increased fork stalling and breakage is observed at the RFB in both *dna2-2* and in some *sgs1* mutants, and is Fob1 dependent.[132] Increased Holliday junction intermediates are also observed at the RFB in *dna2-2* and *sgs1* mutants,[131] and in human cells deficient in WRN.[113,133] Replication fork stalling may result in more secondary structures at 5′ flaps, and might also allow 5′ flaps to equilibrate to 3′ flaps. Dna2 or Sgs1 helicase may be required to process 3′ flaps, or flaps with secondary structure. Stalled forks may also be converted to DSBs, and Dna2 and Sgs1 may function together in their repair.[90,91]

5.5.2 Sgs1 Resolves Holliday Junctions at Stalled Forks

SGS1 was originally identified as a suppressor of the slow growth phenotype of *top3* mutants.[116] Both BLM and Sgs1 interact functionally and physically with topoisomerase 3, a type I topoisomerase that relaxes negatively supercoiled DNA.[134] The Sgs1/Top3 interaction depends on the N-terminal 158 amino acids of Sgs1.[135] The role of BLM or Sgs1 and Top3 is most probably that of an anti-recombinase that dissolves double Holliday structures.[112,136] Both *sgs1* and *top3* mutants show hyper-recombination in the rDNA and in chromosomal duplicated sites.[115,116] Sgs1 and Top3 function together to disrupt double Holliday structures.[136] Top3 resolves the structures, most probably hemicatenanes, created by Sgs1, that are likely to be toxic if not resolved. Interestingly, the viability of the *top3* mutant depends on Pif1. Overexpression of Pif1 but not Rrm3 suppresses the slow growth phenotype of *top3* mutants.[137] In addition, the *top3Δ pif1Δ* combination is lethal; the lethality is suppressed by *sgs1* mutation, since *sgs1 top3 pif1* yeast cells are viable.[137] Therefore, Pif1 can inefficiently resolve structures created by Sgs1, if Top3 is absent. Since Pif1 is a 5′ to 3′ helicase and Sgs1 is a 3′ to 5′ helicase, they could be working in opposition. Unlike in *S. cerevisae*, *top3Δ* mutants are lethal in *S. pombe*. This lethality is suppressed by deletion of the homologue of the *SGS1* gene, *rqh1*$^{+}$.[138,139] The Pif1 homologue in *S. pombe*, Pfh1, appears not to suppress *top3* lethality.[51] Therefore, in the case of suppression of *top3* lethality in *S. pombe*, Pfh1 functions like *S. cerevisiae* Rrm3 instead of like Pif1.

Is it possible to separate the Holliday junction dissolving function of Sgs1 and BLM, which causes hyper sister chromatid exchange in BS cells, from their role in DNA replication and OFP? *sgs1(Δ200)* with a deletion of 200 amino acids comprising the conserved HRDC (helicase and RNaseD C-terminal) domain of BLM but retaining the RQC domain, acts like a separation of function mutation in this sense. The HRDC domain is required for BLM and Top3 together to dissolve double Holliday junctions *in vitro*, but is not required

for BLM binding to a DNA forked substrate, G4 DNA structures, or for its helicase activity.[140] Thus, it represents a second DNA binding domain in addition to the RQC.

The HRDC domain has also been implicated in the strand annealing and strand exchange activities of BLM.[140] Unlike *sgs1Δ* and *sgs1-RQC* mutants, *sgs1(Δ200)* mutants are resistant to MMS and do not exhibit hyper-recombination at chromosomal duplicated sites, a phenotype putatively attributable to defective replication.[128] However, *sgs1(Δ200)* mutants are defective in a phenotype probably involving the resolution of double Holliday structures, *i.e.* the suppression of the normal growth rate of *top3Δ sgs1Δ* mutants. *top3Δ* strains grow slowly; *top3Δ sgs1Δ* strains grow faster, *top3Δ sgs1Δ* transformed with a *CEN SGS1* plasmid grow slowly, but *top3Δ sgs1Δ* strains transformed with *sgs1(Δ200)* grow like *top3Δ sgs1Δ* strains.[128] One interpretation is that Sgs1(Δ200) is not creating the substrate for Top3, presumably a hemi-catenane derived from a double Holliday structure. In addition, *sgs1(Δ200),* unlike *sgs1Δ*, is not synthetically lethal with *mms4* (the partner to nuclease Mus81), which may be involved in DNA replication. In addition to the phenotypes of *sgs1(Δ200),* BLM with a deletion of the HRDC domain complements the temperature sensitivity of a *dna2-1* mutation, while deletion of RQC does not, also suggesting a separation of the DNA replication and Holliday-resolving functions of BLM, and by analogy Sgs1 (L. Liu and J. L. Campbell, unpublished data). Finally, BLM lacking the HRDC domain does not prevent the hyper-sister chromatid recombination phenotype of BS cells.[141]

5.5.3 Mus81 Nuclease in OFP and Stalled Fork Resolution

Mus81/Mms4 is a structure-specific endonuclease that cleaves 3′ flaps.[142] *mus81Δ* is represented in the network shown in Figure 5.1 and is synthetically lethal with *sgs1Δ,* and *sgs1Δ mus81Δ* synthetic lethality is partially suppressed by *rad51Δ*.[143] The triple *sgs1Δ mus81Δ rad51Δ* mutant grows significantly more slowly than *sgs1Δ rad51Δ*. This suggests a complex set of functions in both replication and recombinational repair.

A role for *Mus81* in replication is more directly suggested by the fact that *rnh202*, encoding a subunit of RNaseH2, is synthetically lethal with the *sgs1Δ mus81Δ rad51Δ* triple mutants.[144,145] Mus81/Mms4 thus appears to compensate for the absence of RNaseH removal of RNA/DNA hybrids during OFP. One possible role of Mus81 in OFP could be processing 3′ flaps generated by branch migration of 5′ flaps (equilibration of flaps) at stalled forks whose processing is delayed or impaired. This is consistent with *mus81Δ rad27Δ* synthetic lethality.[146,147] Long 3′ flaps are not expected to form when pol δ is bound to the 3′ OH of the growing Okazaki fragments, *i.e.* without dissociation of pol δ. Such dissociation may occur when forks are stalled, however, such as at the ribosomal RFB, in replication slow zones, or at repeated DNA sequences. If Mus81 and Sgs1 are processing stalled reversed 3′ flaps at the RFB then the triple *mus81Δ sgs1Δ fob1Δ* might be viable. Notably in *S. pombe*,

Mus81 and the RecQ homologue, Rqh1, act to process stalled replication forks prior to their regression into Holliday junctions.[148]

In meiotic cells, Sgs1 functions as an antirecombinase; it is possible that it may do so also in mitotic cells. Sgs1 localizes to and disrupts sites of crossing over.[149] In *sgs1* mutants, the outcome of undisrupted Holliday junctions is joint molecules involving three and four chromosomes.[150] Meiotic cells missing both Sgs1 and Mus81/Mms4 thus accumulate highly elevated levels of joint molecules, which persist through meiosis and are inefficiently processed to crossovers. Expression of either Sgs1 or Mus81/Mms4 during meiosis in the *sgs1 mus81* strain allows dissection of their contribution to crossover formation: Sgs1 prevented formation of joint molecules, whereas Mus81 did not prevent joint molecule formation but allowed resolution of the joint molecules into crossovers.[150–152]

5.5.4 RecQ Proteins Stabilise Stalled Replication Forks

In addition to resolution of structures arising at collapsed or broken replication forks, Sgs1 may be able to prevent fork collapse. A role has been proposed for Sgs1 in the S phase checkpoint, based on observations that Rad53 phosphorylation is slightly defective in an *sgs1* mutant after HU treatment.[120] This is supported by the fact that in *sgs1Δ rad24Δ* strains, phosphorylation of Rad53 after HU treatment is nearly blocked, showing that Sgs1 and Rad24 function in parallel checkpoint pathways (yeast Rad24 is a component of the DNA damage checkpoint pathway important for loading the PCNA-like repair sliding clamp 9-1-1[153] (Rad17-Mec3-Ddc1 in yeast; see Section 3.5).

The role of Sgs1 in Rad53 activation probably involves Sgs1/Rad53 protein–protein interaction and cellular co-localization at the DNA replication fork; human WRN and BLM also co-localise to sites of DNA replication.[121,122,154] However, the sensitivity of the *sgs1Δ* strains to HU is not due to an inability to activate Rad53. Instead, the HU sensitivity may be due to reduced stability of pol ε and pol α at stalled replication forks in *sgs1Δ* mutants.[155] The HRDC domain of Sgs1 is not required for stable maintenance of pol ε at HU-stalled replication forks[156] suggesting that Sgs1 does not need antirecombinase activity to stabilise stalled forks. The enhanced fork arrest observed in *sgs1Δ* strains at the ribosomal replication fork barrier (see Section 5.5.1) might result from the reduced stability of pol ε and pol α at the RFB. Pol ε is also unstable in HU-arrested *mec1* strains, and a combined *mec1 sgs1* mutation increases this instability. These observations on polymerase stability correlate with survival, since double mutant *sgs1 mec1-100* strains show synergistic reduction in survival after HU treatment, compared with single mutant *sgs1* or *mec1-100* strains.[157] The *mec1-100* mutant is resistant to MMS, but does not delay late origin firing in the presence of MMS.[158,159] In a strain with the *mec1-100* mutation, Sgs1 is required to maintain RPA at HU-stalled replication forks. The DNA structures that bind RPA are probably unstable in *sgs1 mec1-100* mutants. Thus Sgs1 may be important in stabilising stalled replication forks, so

that restart is rapid and will not require re-licensing or replication from forks emanating from distant origins. In human cells, BLM is implicated in replication fork restart;[160] the finding of WRN at two-thirds of all replication foci[122] and of abnormal fork asymmetry in ~70% of origins in cells lacking WRN[114] supports the importance of RecQ proteins in stabilizing and promoting restart of stalled replication forks.[108,114,133]

Gross chromosomal rearrangements (GCR; *e.g.* deletions, insertions, translocations and telomere additions) are thought to occur at chromosome fragile sites when replication forks stall or fail, and there is a high degree of redundancy in the mechanisms that suppress these rearrangements. Interestingly, the putative replication fork disruptions due to Sgs1 insufficiency correlate with an increased GCR rate in *sgs1, mec1, rad53* and *rad24* strains: *sgs1Δ rad24Δ, sgs1Δ rad53,* and *sgs1Δ mec1* strains show synergistic GCR rates compared with the single mutants.[157,161] The GCR rate of the *sgs1* mutant is similar to that of the *dna2-2* mutant, while that of the *mre11* mutant strain is about 30-fold greater than in the *sgs1* mutant, and the *sgs1 mre11* rate is similar to the single *mre11* mutant.[162]

5.5.5 Implications for Understanding Genome Instability in Human Disease

Does research on BLM, Sgs1, WRN, Dna2, Rad27 and Mus81 provide any answers as to why Bloom and Werner syndrome patients are so highly cancer prone? The answer is unlikely to be simple. One idea is based on the hyper-recombination phenotype of BS and WS cells. Suppose cells are heterozygous for numerous tumour suppressor genes (TSG), in that inactivation of the one active copy of any particular TSG will not promote neoplastic change. However, when many of the TSGs are sequentially or coordinately inactivated, then cancer will almost certainly result. The error rate of DNA replication is 10^{-10} per base pair (Chapter 4), so that TSG inactivation resulting from replication is likely to be rare. However, if BS or WS cells are defective in OFP or restart of stalled replication forks, even if only mildly, then the BLM/WRN mutation might not only raise the overall error rate of replication but also amplify each error very quickly. Thus BLM/WRN mutation would greatly accelerate loss of heterozygosity of the TSGs and lead to high cancer incidence, as is observed in the clinic.[163]

5.6 Nucleases and Helicases in Telomere Maintenance

Telomeres represent chromosomal ends that are, like DSBs, processed by nucleases and helicases, but the processing mechanism is different, unless a telomere becomes a DSB on loss of its protective cap or end binding protein Cdc13.[164] Telomere length is controlled by addition of G/T rich repeats at the ends of chromosomes by telomerase. This is regulated by telomere binding

proteins and the OFP processing machinery, which is required to convert the telomerase product into duplex form (see Chapter 8).

5.6.1 Recruitment of Telomerase to the Telomere

In late S phase of *S. cerevisiae*, Est1, a subunit of telomerase, is recruited to telomeres by a pathway requiring Mre11 and Tel1.[165] Tlc1 (telomerase RNA), pol α and pol δ are also required for *de novo* telomere synthesis.[166] This reaction is blocked in cells with a C-terminal deletion mutant of Xrs2 protein that cannot bind Tel1.

Mre11 is thought to create the G/T 3′ telomere overhang strands that are present constitutively throughout the cell cycle, which lengthen in late S phase[167] (the specific function of which in telomere maintenance is not clear), since *mre11Δ* strains have reduced levels of single-stranded GT overhangs[166] and telomeres that are 150 bp shorter than in *MRE11* strains.[168] A role for Mre11 nuclease in telomere addition is further supported by the finding that the X-ray resistant *mre11* mutant, *mre11-3* (H125L/D126V), which is presumed to be nuclease deficient, has normal length telomeres but is blocked in *de novo* telomere synthesis in G2 arrested cells.[169,170] However, the amount of single-stranded GT overhang DNA and overall telomere lengths of *mre11-D56N* and *mre11-H125N,* strains deficient specifically in the nuclease activity, are the same as in wild-type *MRE11* strains.[165] The 5′ to 3′ nuclease digestion that is mediated by MRX and observed in late S phase may therefore not be required for telomere synthesis, but for some other aspect of telomere maintenance. This leaves open the role of Mre11 nuclease in telomere biosynthesis,[171] especially as Mre11 does not appear to be the sole nuclease capable of either creation of the single-strand G/T overhangs in late S phase or telomere length regulation. Interestingly, 5′ to 3′ nuclease digestion of the 5′ C/A strand is not observed during *de novo* telomere synthesis.[170]

5.6.2 Dna2, Exo1, and Sgs1 in Telomere Processing

The apparently normal telomeres in nuclease-deficient Mre11 mutants may arise, at least in part, from the existence of a nuclease that compensates for Mre11 nuclease but not its other functions; the identity of this putative nuclease is unknown. ExoI is a candidate since it plays roles at telomeres under some, but not all, conditions. *exo1Δ* causes no change in telomere length nor does overproduction of Exo1 lengthen telomeres in a *mre11Δ* strain.[82] Therefore Exo1 is not sufficient to compensate for Mre11 in telomere maintenance, and presumably another nuclease compensates. However, when telomeres are uncapped by Cdc13 inactivation, then Exo1 is the major player in 5′ to 3′ degradation.[172,173] In this case the dysfunctional telomere may resemble a DSB, where Exo1 is thought to play a role, at least in extensive resection.

A role also for Dna2 at telomeres is suggested by several lines of evidence. Firstly, *dna2-2* mutants are defective in *de novo* telomere synthesis:[174] the gross

chromosomal rearrangement (GCR) rate of the *dna2-2* strain is about 20-fold greater than wild type. Secondly, telomeres of *dna2Δ pif1Δ* mutants are shorter than *pif1Δ* mutants at 30 °C.[44] Moreover, Dna2 protein associates with telomeres during G1, moves to bulk chromosomal DNA during S phase, and then re-associates with telomeres during G2,[174] but is released from telomeres in the presence of DNA damage.

In this context, it is of note that Dna2 not only binds with high affinity to G4 DNA (found at telomeres) but that it can also unwind such structures via its helicase activity.[27] Similarly, Mre11 binds G4 DNA, and shows weak nuclease activity on this template.[175] The G4 binding activity of Dna2 and Mre11 may be involved in telomere synthesis, but their relative importance may depend on Pif1, and any possible G4 binding/unwinding by this helicase. For example, the GCR rate of a *dna2Δ pif1-m2* strain is about half that of the *pif1-m2* strain, defective in nuclear Pif1, suggesting the Dna2 is required for telomere addition.[44] Interestingly, *dna2-2 est2* strains die shortly after telomerase loss, with survivors relying on telomere GT recombination (Type II) rather than subtelomeric Y′ repeat recombination (Type I) for their survival.[44,174] The significance of these observations is the subject of active study.

Telomere maintenance in the absence of telomerase, which occurs through a recombinational pathway between telomeric GT repeats (Type II), also requires the action of the Sgs1 helicase;[176,177] Sgs1 has G4 binding and unwinding activity, and in telomerase-negative (*est2*) cells, absence of Sgs1 leads to rapid telomere-driven senescence.[178] It is of note that again Mre11 is important, since both Mre11 and Sgs1 are required for the recombination between telomere GT repeats observed in telomerase-minus cells.

5.6.3 Preferential Elongation of Short Telomeres

Short telomeres can activate the DNA damage checkpoint, resulting in cellular senescence or apoptosis. Probably to avoid this, short telomeres are preferentially elongated; yeast telomeres of length 300 bp have an 8% chance of being elongated per cell cycle, while a decrease in length to 100 bp increases the frequency to 46%.[179] In *S. cerevisiae,* telomere length is regulated by the Rap1/Rif1/Rif2 complex. Increased numbers of telomere GT repeats (which depends on Mre11—see Section 5.6.2) result in increased Rap1 binding. Rap1 then binds Rif1 and Rif2, which inhibit elongation by telomerase.[180]

To examine the mechanism of preferential elongation of short telomeres, a chromosome was engineered in which a short telomere can be created by site-specific recombination.[181] Shortening of the telomere resulted in enhanced binding of telomerase components Est1 and Est2, but not the capping protein Cdc13. Cdc13 binding to telomeres is similar in wild type, *mre11Δ* and *tel1Δ* yeast cells,[182] suggesting that Cdc13 recruitment is independent of both Mre11 and Tel1. By contrast, increased binding of Est1 and Est2 to telomeres has also been observed in S phase, and this enhanced binding is dependent on Tel1.[183]

A major role of MRX in telomere elongation is in recruiting Tel1 to telomeres in late S phase; *tel1*Δ strains have short telomeres. This can be corrected by galactose-induced overproduction of Tel1 in a *tel1*Δ strain, resulting in Mre11-dependent Rad53 phosphorylation which persists for 12 hours and occurs concurrently with telomere elongation,[184] supporting the importance of Tel1 in telomere elongation. Rad53 phosphorylation lasts much longer when Tel1 is overproduced in a *sae2*Δ or *rad50S* mutant;[97] however the telomeres remain short, perhaps reflecting the requirement for Mre11 nuclease in the *de novo* generation of telomeres after a DSB. The time course of Rad53 phosphorylation upon Tel1 overproduction correlates with MRX binding to telomere ends in wild type, *sae2*Δ and *rad50S* strains. The enhanced binding of Tel1 and Est2 to short telomeres is dependent on MRX, and occurs with reduced Rif2 but not Rif1 binding. Overproduction of Rif2 at the same time as overproduction of Tel1 inhibited both MRX binding to telomere ends and Rad53 phosphorylation, however, suggesting that the Rif2 'counting mechanism' involves inhibition of MRX binding to the telomere.[180,184] Creating a strain with a 90 bp telomere activated MRX-dependent Rad53 phosphorylation over a 24 hour time period, correlating MRX telomere binding and telomere elongation with Rad53 phosphorylation.[184] These data show that in yeast, as in mammalian cells, short telomeres can activate the DNA damage checkpoint, although it is unlikely that telomeres as short as 100 bp occur naturally in wild-type *S. cerevisiae*.

5.6.4 Inhibition of Telomerase by Helicases

Pif1 helicase inhibits telomerase and *pif1Δ* strains have long telomeres.[45,46] The telomere lengths in *MRE11pif1Δ* and *mre11Δ pif1Δ* strains are closer in size than in *MRE11* and *mre11Δ* strains[185] (M. E. Budd and J. L. Campbell, unpublished data), which could suggest that an additional role of Mre11 in telomere synthesis is to suppress Pif1 inhibition of telomerase, and that the nuclease active site of Mre11 might be required for this suppression. This idea is testable by examining *de novo* telomere synthesis in *pifΔ1* and *mre11Δ pif1Δ* and in *mre11-nd pif1Δ* strains, and is supported by data from gross chromosomal rearrangement (GCR) assays developed by Kolodner and collaborators. The GCR selection involves marking a chromosome with *CAN1* and *URA3*, and assaying for chromosomal breakage and repair by quantifying canavanine resistant (*canR*) and 5-FOA resistant colonies.[9] Survivors are characterized by DNA sequencing, which reveals that repair can occur by telomere addition, micro-homology joining or translocation, as well as determining the frequency of each event. As there is no telomere seed sequence at the presumed DSBs initiating the rearrangements, telomere addition is likely to be inefficient in these assays. In wild-type cells, GCR survivors (*canR* and FOA resistant) almost always show telomere additions.[186] *mre11* mutation increases the GCR rate by 600-fold, and only a minority (30%) of the GCR survivors had telomere additions.[187] *mre11Δ pif1Δ* strains, on the other hand, have about a four-fold

increased GCR rate relative to *mre11Δ*, but the survivors all showed telomere additions.[185] The *mre11-H125N* mutation increases the GCR rate by 150-fold compared with wild type, and none of the GCR survivors in the *mre11-H125N* strain had telomere additions, showing a requirement for a nuclease active site in telomere additions. By contrast, the GCR survivors of the *mre11-H125N pif1-m2* mutant are all telomere additions. Thus, inactivation of Pif1 eliminates the requirement for the Mre11 nuclease in telomere addition-type GCRs. These results suggest an interaction of the Mre11 nuclease with Pif1 helicase at telomeres.

5.7 Perspective

S. cerevisiae does not require all the nucleases and helicases involved in genome stability for survival because the size of the genome is small and not all damaged DNA replication forks need to be repaired correctly for survival, since replication is possible from a distal fork in a reasonable time frame. However, the ability to process telomeres and repair endogenous DNA damage resulting from stalled forks is compromised when such factors are missing. In order to increase the size of the genome ten, a hundred or even a thousand fold (as in the human genome), orthologues of *MRE11, SAE2, SGS1, PIF1, RAD27*, *DNA2, EXO1* and *MUS81/MMS4* all become essential to correct the inevitable damage (*e.g.* single- and double-strand breaks and partially processed Okazaki fragments) created by a moving DNA replication fork. An estimate of the number of endogenous DSBs created per cell per division in human cells is between 12 and 50;[188,189] none of these should be left unrepaired, otherwise cell death or neoplastic change leading to cancer remain alternative fates. By utilising a network of interacting helicases and nucleases to maintain genome stability, fungi anticipated the evolution of large complex genomes coding for long-lived complex organisms. An extremely useful outcome of this is the ability to study replication proteins in the simple yeast system, with the confidence that their roles will be highly conserved in higher organisms.

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